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Synthesis and Evaluation of Luminescent Tracers and Hapten-Protein Conjugates for Use in Luminescence Immunoassays with Immobilised Antibodies and Antigens

A critical study of macro solid phases for use in immunoassay systems, Part II[1])

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Summary: This article describes the synthesis of labels and hapten-protein conjugates for use in bio- and chemiluminescent immunoassay systems, together with the problems encountered.

The effects of maleimide upon acetate-, adenylate- and pyruvate kinase activity have been studied, as well as upon the luciferin-luciferase monitoring system. Maleimide inhibited both acetate and adenylate kinase but showed no inhibition of pyruvate kinase and the monitoring reagent.

Four heterobifunctional reagents were tested for their capability in forming pyruvate kinase-donkey-anti-rabbit IgG conjugates which retained enzyme and antibody activity. The best results were obtained with succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate and succinimidyl-6-(*p*-maleimidophenyl)-hexanoate.

The relationship between the amounts of succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate and IgG was studied with respect to enzymic activity of the conjugate.

The *Michaelis-Menten* constants for both conjugated and non-conjugated pyruvate kinase were calculated and compared. It was found that the maximal velocity (V_{max}) of the conjugated enzyme was lower than that of the non-conjugated enzyme although the "apparent" K_m value was the same for both conjugated and non-conjugated pyruvate kinase.

The pyruvate kinase-anti rabbit IgG conjugate was tested for its ability to bind to rabbit-IgG coated polystyrene balls.

In addition to bioluminescent labels, the synthesis of chemiluminescent markers was undertaken and optimised. The three substances used for labelling were diazoluminol, diazoisoluminol and N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide the latter being used as an N-hydroxysuccinamide "active" ester.

The ratio of label to IgG was studied for diazoluminol and N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester after it had been discovered that diazoisoluminol was not suitable for coupling to antibodies. The optimal molar ratios label: IgG were for diazoluminol 40:1 and for N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester 60:1. Increasing the substitution rate led to a lessening of the dynamic range, shown by an increase in the ratio between unspecific binding (noise) to maximal binding (signal) in an assay.

The synthesis of hapten-protein conjugates for covalent coupling to polystyrene balls was undertaken as this formed part of the preparation for the assays described in Part III. The optimal production of gentamicin-bovine serum albumin and thyroxine-transferrin conjugates has been described in detail.

[1]) Part I: J. Clin. Chem. Clin. Biochem. *21*, 789–797. Part III: J. Clin. Chem. Clin. Biochem. *22*, 349–356.

Synthese und Beurteilung von Lumineszenz-Markern und Hapten-Protein-Konjugaten für Lumineszenz-Immunoassays mit immobilisierten Antikörpern und Antigenen.
Eine kritische Untersuchung von Makro-Festphasen zum Gebrauch in Immunoassay-Systemen, Teil II[1])

Zusammenfassung: Dieser Beitrag beschreibt die Herstellung von Markern für die Verwendung in Bio- und Chemilumineszenz-Immunoassays und zeigt die dabei bestehenden Probleme auf.

Der Effekt von Maleinimid auf Acetat-, Pyruvat- und Adenylat(Myo)kinase zur Klärung ihrer Verwendbarkeit als Biolumineszenzmarker wurde untersucht. Lediglich bei der Pyruvatkinase wurde keine Hemmung beobachtet, das Luciferin-Luciferase-System (Indikator-Reagenz) blieb ebenfalls unbeeinflußt.

Vier heterobifunktionelle Reagenzien wurden auf ihre Eignung für die Synthese von Esel-anti-Kaninchen IgG-Pyruvatkinase-Konjugaten untersucht. Die besten Konjugate wurden mit Succinimidyl-4-(N-maleimidomethyl)-cyclohexan-1-carboxylat und Succinimidyl-6-(*p*-maleimidophenyl)-hexanoat erzielt.

Das molare Verhältnis von Succinimidyl-4-(N-maleimidomethyl)-cyclohexan-1-carboxylat zu IgG wurde im Hinblick auf die Beeinflussung der enzymatischen Aktivität ihres Konjugates mit Pyruvatkinase untersucht.

Die *Michaelis-Menten* Konstanten für konjugierte sowie unkonjugierte Pyruvatkinase wurden bestimmt und gegenübergestellt. Dabei konnte eine Abnahme der maximalen Reaktionsgeschwindigkeit des an IgG gekoppelten Enzyms nachgewiesen werden. Es ergab sich dabei ein „apparenter" K_m-Wert, der dem ungebundener Pyruvatkinase entsprach.

Die Bindungsfähigkeit der Pyruvatkinase-Esel-anti-Kaninchen-IgG-Komplexe an mit Kaninchen-IgG beschichtete Polystyrolkugeln wurde untersucht und dokumentiert.

Darüberhinaus wurde die Synthese von Chemilumineszenz-Konjugaten untersucht und optimiert. Die Eignung von Diazoluminol, Diazoisoluminol sowie N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamid (aktiver Ester) zur Kopplung an IgG wurde untersucht und die optimalen Verhältnisse zu IgG erarbeitet. Diese betragen für Diazoluminol zu IgG etwa 40:1 und für N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamid (aktiver Ester) zu IgG etwa 60:1. Diazoisoluminol war nicht zur Kopplung an IgG verwendbar. Höhere Substitutionsraten führten für Diazoluminol zu einem Anstieg der unspezifischen Bindung bei gleichzeitiger Erniedrigung der spezifischen Bindung. Im Falle von N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamid (aktiver Ester) veränderte sich das Verhältnis von unspezifischer zu spezifischer Bindung trotz höherer Substitution nur unwesentlich.

Die Synthese von Hapten-Protein-Konjugaten zur kovalenten Kopplung an Polystyrolkugeln wurde durchgeführt, da sie Teil der Vorbereitung für die in Teil III beschriebenen Assays ist. Die optimierte Herstellung von Gentamycin-Rinderserumalbumin- und Thyroxin-Transferrin-Konjugaten ist im Detail beschrieben.

Introduction

There are two distinct types of label used in luminescence immonoassays, which are usually referred to as chemiluminescent and bioluminescent marker substances. The difference as far as the assay technique itself is concerned, is only in the way in which the light is produced at the end of all reaction steps.

Labels commonly used in bioluminescence immunoassays are kinases (1, 2, 3) and NAD(P)-dependent dehydrogenases (4, 5).

Difficulties arise in stabilising bioluminescent labels in liquid form however, as the enzymes present are inhibited by conventional antimicrobial agents such as azide or merthiolate.

Chemiluminescent labels include derivatives of isoluminol (6, 7, 8), acridinium esters (9), azoluminol (10) and fluorescein isothiocyanate (11).

Useful labels examined in this study are pyruvate kinase (EC 2.7.1.40) as bioluminescence-(1–3), diazoluminol (10) and N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester as chemiluminescence labels.

The bioluminescent system is based on an ATP-generating system coupled to a firefly luciferin-luciferase light generating reaction (1–3). A species-specific antibody is labelled, which results in the formation of a pyruvate kinase-immunoglobulin G conjugate.

Coupling reactions have been tested using different heterobifunctional reagents (1, 12) having a maleimide and N-hydroxysuccinimide functional group for coupling via thiol and aminogroups respectively. This type of reagent is far superior to alternative reagents such as pentane-1,5-dial, carbodiimides or mixed anhydride reactions. The reaction is a two-step one, the first being the aminoacylation of the IgG followed by the formation of a thioether with the pyruvate kinase.

The optimisation of signal to noise ratio in terms of the maximum and unspecific binding has been studied in terms of reagent and reactant purity and degree of substitution of the label.

The somewhat complicated synthesis of N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester (8) described in the literature was replaced by a simpler method which could be performed in almost any laboratory.

Tab. 1. Reactions catalysed by the kinases used in this study. All reactions were "driven" in the direction of ATP formation.

1. Pyruvate kinase – EC 2.7.1.40 (from rabbit muscle)
   Phosphoenolpyruvate + ADP = Pyruvate + ATP
   Cofactors Mg^{+2} and K^+
   pH optimum – 7.0

2. Acetate kinase – EC 2.7.2.1 (from *E. Coli*)
   Acetyl phosphate + ADP = Acetate + ATP
   Cofactors probably Mg^{+2} or Mn^{+2}
   pH optimum – 7.4

3. Adenylate kinase – EC 2.7.4.3 (from rabbit muscle)
   2 ADP = ATP + AMP
   Cofactor Mg^{+2}
   pH optimum – 6.7

Explanations:

Adenylate kinase is also known as myokinase.
All reactions could be run in the reverse direction as only an exchange of high energy phosphate bonds took place.

Materials and Methods

Materials

Unless otherwise stated, materials were obtained from the same sources as in the first part of this publication (14). Pyruvate kinase, acetate kinase and adenylate kinase (myokinase) were purchased from Boehringer-Mannheim, Mannheim, D.

All heterobifunctional reagents were obtained from Sigma, Munich, D, except for the *n*-hexanoyl derivative, which was a gift from Boehringer-Mannheim.

ATP-monitoring kits and N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide were bought from LKB, Munich, as were the gels Ultrogel A4 and A6.

Glutathione (reduced form), 5,5'-dithiobis(2-nitrobenzoic acid), adenosine diphosphate (ADP) and phosphoenolpyruvate were obtained from Sigma, Munich.

Buffer substances were purchased from Merck, Darmstadt, D., and from EGA-Chemie, (Aldrich) Steinbach, D.

Methods

Effects of maleimide upon monitoring reagent and pyruvate-, acetate- and adenylate kinase activity

All enzyme reactions were run in the "reverse" direction, synthesising adenosine triphosphate (ATP) from the relevant components. The enzyme reactions are shown in Table 1.

First the effect of maleimide upon the ATP-monitoring reagent was studied, to check for inhibition of light production in the luciferin-luciferase system.

Increasing amounts of maleimide (covering the range 0.1–125 mmol/tube) were added to suitable dilutions of the enzymes in incubation buffer (0.05 mol/l Tris-HCl containing 2.5 g/l bovine serum albumin and 0.1 mol/l KCl adjusted to pH 7.4), the effects on the rate of ATP synthesis being plotted against time. ATP production was measured kinetically using an ATP-monitoring kit and an LKB 1250 Luminometer with printer display (see section "*Studies upon the effect . . .*" for detailed information).

Synthesis of the pyruvate kinase IgG conjugates

The γ-globulin fraction of a donkey anti-rabbit IgG serum (Wellcome RD 17) was prepared using polyethylene glycol precipitation followed by DEAE-cellulose column chromatography, (2, 15). The IgG concentration was around 15 g/l in the eluate.

To a solution of IgG (15 g/l) containing a total of 15 mg dissolved in 1 ml buffer 2 (0.02 mol/l potassium phosphate containing 0.15 mol/l NaCl, adjusted to pH 7) was added 1 ml of the respective heterobifunctional reagent (for structure of these reagents see fig. 1.) representing 1 μmol–10 μmol (see section "*Studies upon the effect . . .*" below). The mixture was incubated for 45 min at ambient temperature and then dialysed twice for 1 h against 1 l buffer 2. The degree of coupling was determined by the titration of the free thiol groups on the IgG-heterobifunctional reagent complex with *Ellman*'s reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) in which 2-nitro-5-mercaptobenzoic acid is produced in the presence of free –SH groups. The latter gives an intensive yellow coloured anion which can be measured photometrically at 412 nm (16, 17). A standard curve was set up using reduced glutathione as calibrator covering the range 0–50 nmol/tube.

In order to obtain a label with a higher molar ratio pyruvate kinase: IgG, the pyruvate kinase concentration was held at 4 times that of IgG.

The reaction was allowed to proceed for 25 min at room temperature and was stopped by addition of 100 nmol reduced glutathione. The mixture was then separated on an Ultrogel A6 column (100 × 1.8 cm) using buffer 2 as eluent. The antibody binding potency and enzyme activity were tested in each fraction, the optimal fractions showing both enzyme activity and specific antibody binding pooled, portioned and lyophilised after addition of 1 mg lactose to each portion. The pyruvate kinase activity was tested for as already described (1), the antibody binding potency by binding studies using rabbit IgG covalently coupled to polystyrene balls (14).

Studies upon the effect of heterobifunctional reagent concentration with respect to enzyme activity

The effect upon enzyme activity after coupling to IgG was studied using three different concentrations of succinimidyl-4-(N-male-

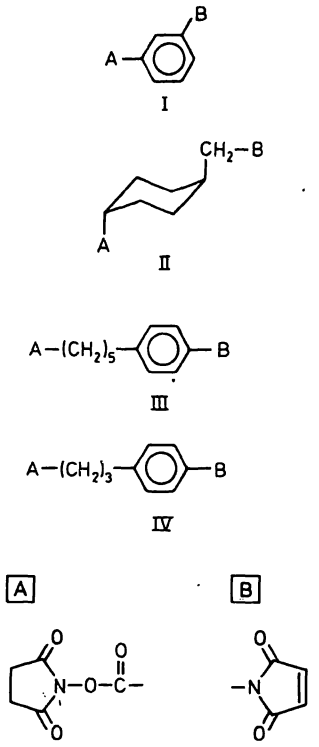

Fig. 1. The structure of the four heterobifunctional reagents used in this study:
I *m*-maleimidobenzoyl N-hydroxysuccinimide ester
II succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate
III succinimidyl-6-(*p*-maleimidophenyl)-hexanoate
IV succinimidyl-4-(*p*-maleimidophenyl)-butyrate
The structures A (N-hydroxysuccinimide-ester group) and B (maleimide group) shown in the lower portion of the diagram are the two functional groups common to the reagents represented here.

imidomethyl)-cyclohexane-1-carboxylate, with a molar ratio IgG: succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate of 1:1.2, 1:6 and and 1:10.

Conjugates were made as in section above, but with additional purification after the succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate-IgG coupling using a Sephadex G-25 column (30 × 1.5 cm). Pyruvate kinase was allowed to react in a four-fold excess, and the optimal fractions (see above) used to calculate the *Michaelis-Menten* constant. The necessary experiments were carried out as set out below:

100 µl of a 1:600 dilution of the conjugate (ca. 2.8 pg IgG) were reacted with a rabbit IgG-coated polystyrene ball (14) in 200 µl incubation buffer for 3 h at ambient temperature (23 °C). The polystyrene ball was then washed with incubation buffer (2 × 500 µl) and 0.15 mol/l NaCl (1 × 500 µl).

The measurement of ATP production in order to calculate the K_m-value for ADP was carried out using the following system:

200 µl Tris-HCl buffer (0.1 mol/l, pH 7.4)
100 µl ATP monitoring reagent
50 µl Phosphoenolpyruvate (2 g/l in 0.2 mol/l KCl/$MgSO_4$)
50 µl ADP (end concentration/tube 4, 0.8, 0.4, 0.08, 0.04 and 0,008 µmol)

The same system was used to calculate the K_m value for phosphoenolpyruvate. Here the ADP concentration was kept constant at 6 g/l, the concentrations for phosphoenolpyruvate were varied as described above for ADP. The K_m value for "free" pyruvate kinase was measured using a 10 µl aliquot, equivalent to 1 ng/tube.

Lineweaver-Burk plots were made in order to derive the K_m-values for ADP and phosphoenolpyruvate.[f]

Diazotisation of luminol and isoluminol

On account of conflicting reports in the literature (10) and personal experience, the effects of diazotisation time, pH and substitution rate into IgG have been studied. Binding studies have been carried out using the relevant IgG against which the labelled antibody is directed, coupled to polystyrene balls (14).

Luminol or isoluminol (1 mmol) was suspended in 5 ml 1 mol/l HCl and cooled under constant stirring to 0 °C (ice bath). KNO_2 (1.5 mmol) was dissolved in 1 ml distilled water and cooled in ice. The cold KNO_2 solution was added dropwise to the luminol/isoluminol suspension respectively, until the green-yellow slurry became a clear orange (luminol) or yellow-orange (isoluminol) solution. The time taken for the addition of nitrite was 15—20 min. An excess of HNO_2 is usually undesirable if the diazotised reactant is to be used straight away because of further unwanted diazotisation of the second reactant, here IgG. In this case, as IgG molecules contain no arylamino groups, no such side reactions should occur. However, the presence of excess nitrite ions were tested for using starch iodide paper, a blue-black coloration denoting the presence of nitrite. This was removed by adding a few drops of urea solution (1 mol/l) until the reaction with starch-iodide paper was negative. The diazoluminol or diazoisoluminol was used immediately for the preparation of labels. The diazo compounds were stable for periods up to 7 days at −20 °C, but even during this time, their effectiveness as coupling reagents deteriorated. A quick test to see if the diazoluminol was still useable was that if, after addition of a drop of K_2CO_3 (1 mol/l) the red coloration was stable for more than 30 minutes it could still be used for coupling. In the case of diazoisoluminol, the yellow orange solution turned golden yellow and crystallised out inside 30 minutes after beeing synthesised. The resulting suspension was used for conjugation to IgG.

Preparation of diazoluminol- and diazoisoluminol-antibody conjugates

These experiments were carried out to check the effect of pH, time and the ratio of coupling reagent to IgG on the quality of the products formed.

The diazoluminol or diazoisoluminol preparation was added slowly to 1 ml of the IgG solution (15 g/l) in buffer 2 under constant stirring in an ice bath to give final ratios of diazoluminol/diazoisoluminol:IgG of 20:1. The reaction times were 6, 24 and 72 h at pH 4 and pH 9 at 4 °C. Potassium carbonate solution (1 mol/l) was added dropwise to maintain the pH between 9.0 and 9.2. The reactions at pH 4 were carried out maintaining the pH with 0.5 mol/l potassium phosphate buffer pH 7.

After the addition of diazotised (iso)luminol, the reaction was allowed to proceed at 4 °C during which time the reaction mixture changed from red to yellow in case of pH 9 derivatives, those at pH 4 remaining pale yellow throughout the coupling procedure.

After the reaction all reation mixtures were purified over an Ultrogel A4 column (30 × 2.5 cm) with buffer 2 as eluent, collecting 30 drop fractions (1.5 ml). The coloured fractions were tested for specific and unspecific binding to IgG and transferrin coated polystyrene balls respectively (14).

To check the effect of changing the ratio luminogen: IgG diazoluminol was used at pH 9 with a 24 h reaction time at 4 °C as these conditions were optimal.

The ratio of diazoluminol: IgG was varied between 8:1 and 200:1. Separation of the reaction components was made with an Ultrogel A4 (see above).

Optimised preparation of N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester

18.5 mg (50 µmol) N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide were dissolved in 800 µl dry dimethylformamide under slight warming. N-Hydroxysuccinimide (5.75 mg = 50 µmol) was added at room temperature, the mixture being stirred. When the N-hydroxysuccinimide had been dissolved, 30.9 mg (150 µmol) dicyclohexylcarbodiimide dissolved in 200 µl dry dimethylformamide were added. After 20 h at room temperature without stirring and under light exclusion (aluminium foil covered reaction vessel) the mixture was used for direct coupling to antibodies and antigens. During the reaction, crystals of dicyclohexyl urea precipitated out.

A low temperature of about 4 °C slows down the formation of the active ester and an increase in incubation time at room temperature (>20 h) gave no increase in N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester formation.

Product formation was confirmed using thin layer chromatographic techniques. The solvent used was ethyl acetate ethanol NH_3 (25 g/l) 5 + 2 + 1 (by volume). The R_f-values obtained were as follows: N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide – 0.074, N-hydroxysuccinimide – 0.14, N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester – 0.4. Some other spots were observed which demonstrated neither fluorescence when excitated at 366 nm nor luminescence when excited with alkaline microperoxidase and H_2O_2.

Preparation of N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester-antibody conjugates

The γ-globulin fraction of a donkey anti-rabbit IgG serum was prepared as described in Section "*Synthesis* . . .". To 3 × 2 ml (30 mg protein) portions of this preparation different amounts of the N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester preparation were added to give final ratios N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester: IgG of 30:1, 60:1 and 120:1 respectively. After the addition, a precipitate of water insoluble components formed immediately. The reaction was allowed to continue for 24 h at 4 °C under light exclusion. After centrifugation (10 min at 4000 min^{-1}) the supernatant was transferred to an Ultrogel A4 (30 × 2.5 cm) column using 0.05 mol/l tris/HCl pH 8 as eluent, collecting fractions of 1.5 ml.

The test system for specific and unspecific binding was identical with that described in the preparation of diazoluminol and diazoisoluminol-IgG.

Synthesis of a gentamicin-bovine serum albumin conjugate for the gentamicin SPALT assay (see tab. 2)

The coupling reagent used was 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide methyl-*p*-toluene sulphonate (MCDI). The molar ratios chosen were: bovine serum albumin: carbodiimide (MCDI): gentamicin – 1:60:80. Bovine serum albumin (68 mg) and 48 mg gentamicin were dissolved in 4 ml distilled water and the pH adjusted to 4.5 with dropwise addition of 0.1 mol/l HCl. Carbodiimide (MCDI, 28 mg) were dissolved in 500 µl distilled water and added dropwise to the bovine serum albumin-gentamicin mixture. The pH of the resulting mixture was held constant at 4.5 with 0.1 mol/l HCl for the next hour after which the reaction was allowed to proceed at 4 °C overnight.

Seperation of complex and unreacted gentamicin was effected via an Ultrogel A6 column (30 × 2.5 cm) using buffer 2 as elution buffer. The column fractions were tested for protein content ($A_{280\,nm}$) and gentamicin immunoreactivity (radioimmunoassay). From the resulting data it was estimated that an effective (immunoreactive) substitution of 23–28 gentamicin per bovine serum albumin had taken place.

Synthesis of a thyroxine-transferrin conjugate for a T_4-SPALT assay

This conjugate was synthesized for the T_4-SPALT assay in part III of this trilogy and is described here as it is part of the preparation for an assay.

The coupling reagents chosen were 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide methyl-*p*-toluene sulphonate (see above) or 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDAC).

The molar ratios chosen were: Transferrin:carbodiimide(EDAC):thyroxine – 1:50:200.

44 mg transferrin were dissolved in 500 µl distilled water and the pH adjusted to 6 using 0.1 mol/l HCl. 20 mg thyroxine were suspended in 1 ml distilled water and brought into solution by dropwise addition of 0.5 mol/l NaOH. The carbodiimide solution was added dropwise to the transferrin solution keeping the pH at 6, the mixture being allowed to stand for 5 minutes. The thyroxine solution was given dropwise to the mixture over a period of 90–120 minutes, keeping the pH between 6 and 6.5. The mixture was then allowed to stand overnight at 4 °C. The thyroxine did not all remain in solution with 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride as coupling reagent. The high molar ratio of thyroxine was meant to compensate for possible precipitation during the reaction, as it was known that it is almost impossible to keep thyroxine in aqueous solution at pH values lying on the acid side of neutrality.

The purification of the conjugate was via an Ultrogel A6 column (30 × 2.5 cm) using centrifugation step before the column run to remove particulate material.

The substitution rate was determined spectrophotometrically using the molar lineic absorbance for thyroxine at 328 nm as 56 $m^2 \cdot mol^{-1}$.

The substitution rate with 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide methyl-*p*-toluene sulphonate was 16 thyroxine per transferrin and for 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride 24 thyroxine per transferrin.

Results

Bioluminescent conjugates

Figure 2 shows the effect of maleimide on acetate- and adenylate- (or myo-) kinase, figure 3 on the ATP-monitoring reagent.

Table 2 shows the binding of the four pyruvate kinase-IgG conjugates made using the different heterobifunctional reagents

m-maleimidobenzoyl N-hydroxysuccinimide ester,

succinimidyl-4-(*p*-maleimidophenyl)-butyrate,

succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate and

succinimidyl-6-(*p*-maleimidophenyl)-hexanoate,

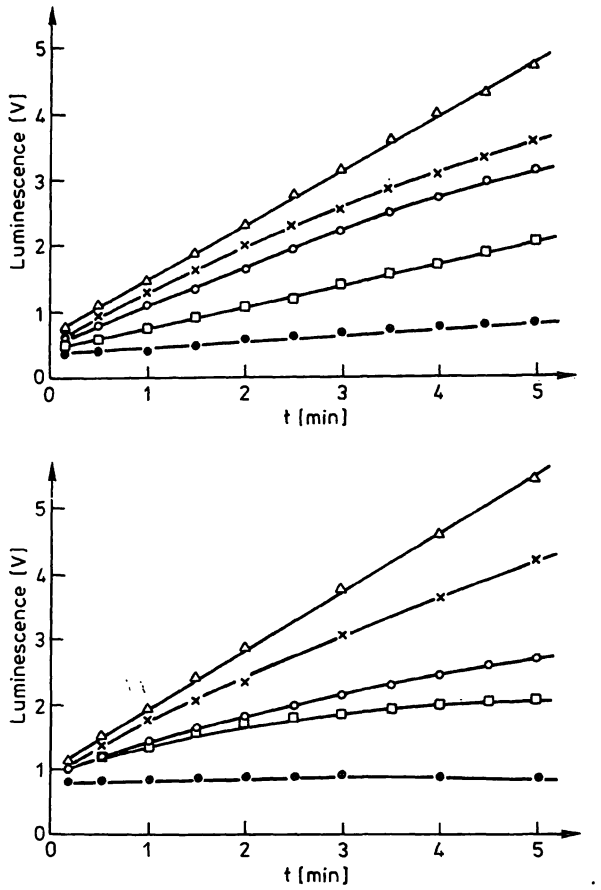

Fig. 2. The effect of maleimide on kinases in terms of concentration and time. The values on the ordinate show the response of the luminometer to ATP-production during the incubation time. A flatter slope corresponds to a lower rate of ATP-production, i.e. a higher degree of inhibition by maleimide.
a) adenylate kinase (myokinase)
b) acetate kinase, using an excess of acetyl phosphate to drive the reaction in the direction of ATP-production.
△—△ no maleimide
x—x 30 mmol/l maleimide
□—□ 3 mmol/l maleimide
○—○ 0.3 mmol/l maleimide
●—● 0.3 mmol/l maleimide, after 30 min

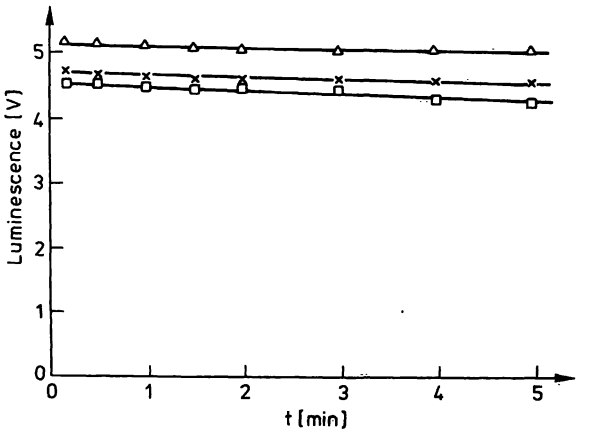

Fig. 3. The effect of maleimide on the ATP-monitoring system (luciferin-luciferase) after injecting ATP ($1 \cdot 10^{-6}$ mol/l = 50 µl).
△—△ no maleimide
x—x 30 mmol/l maleimide
□—□ 3 mmol/l maleimide

Tab. 2. Binding of IgG-pyruvate kinase conjugates prepared by using the heterobifunctional reagents shown in figure 1. The B_0 and UB values are from a gentamicin SPALT assay (3) using polystyrene balls as a support for a gentamicin-bovine serum albumin conjugate (see Materials and Methods section and l.c. (14)).

Dilution of the pyruvate kinase-IgG conjugate 1:40 in incubation buffer. For unspecific binding (UB) bovine serum albumin replaced the gentamicin-bovine serum albumin conjugate on the polystyrene balls (14).

All measurements made on the LKB 1250 luminometer.

Conjugate of donkey anti-rabbit serum and pyruvate kinase with		B_0 (mV/s)	UB (mV/s)
m-Maleimidobenzoyl N-hydroxy-succinimide ester	I	18.10	0.64
Succinimidyl-4-(N-maleimido-methyl)-cyclohexane-1-carboxylate	II	71.83	0.47
Succinimidyl-6-(*p*-maleimido-phenyl)-hexanoate	III	70.33	0.60
Succinimidyl-4-(*p*-maleimido-phenyl)-butyrate	IV	12.25	0.46

The assay conditions were identical to those already published, (3), replacing the microcrystalline cellulose by the antigen-coated polystyrene ball and omitting the centrifugation steps.
Results given as millivolt/second (mV/s) reflecting the rate of ATP production.

to rabbit IgG coated polystyrene balls. All subsequent tests were made with the donkey anti rabbit IgG-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate-pyruvate kinase conjugate as this performed best. Figure 4 shows the *Lineweaver-Burk* plots to test the effect on the enzyme kinetics after coupling pyruvate kinase to IgG. Figure 4a is for ADP, figure 4b for phosphoenolpyruvate.

The results show that the enzyme kinetics are influenced by the coupling. The *Michaelis-Menten* constants for ADP and phosphoenolpyruvate in the antibody-pyruvate kinase conjugates as well as those for unconjugated pyruvate kinase were calculated from figure 4a and 4b. It can be seen that a vertical line connecting the intersection of the antibody conjugate concentrations with the abscissa, goes through the point where unconjugated pyruvate kinase intersects the abscissa. By increasing the amount of succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate to IgG, an increase in reaction rate can be observed, however the maximal velocity (V_{max}) of unconjugated pyruvate kinase is never attained.

The K_m value at 23 °C observed for ADP was 1.74×10^{-4} mol/l (lit. 3.0×10^{-4} at 30 °C (13)) and for phosphoenolpyruvate 2.3×10^{-4} mol/l (lit. 7.0×10^{-5} mol/l at 30 °C (13)).

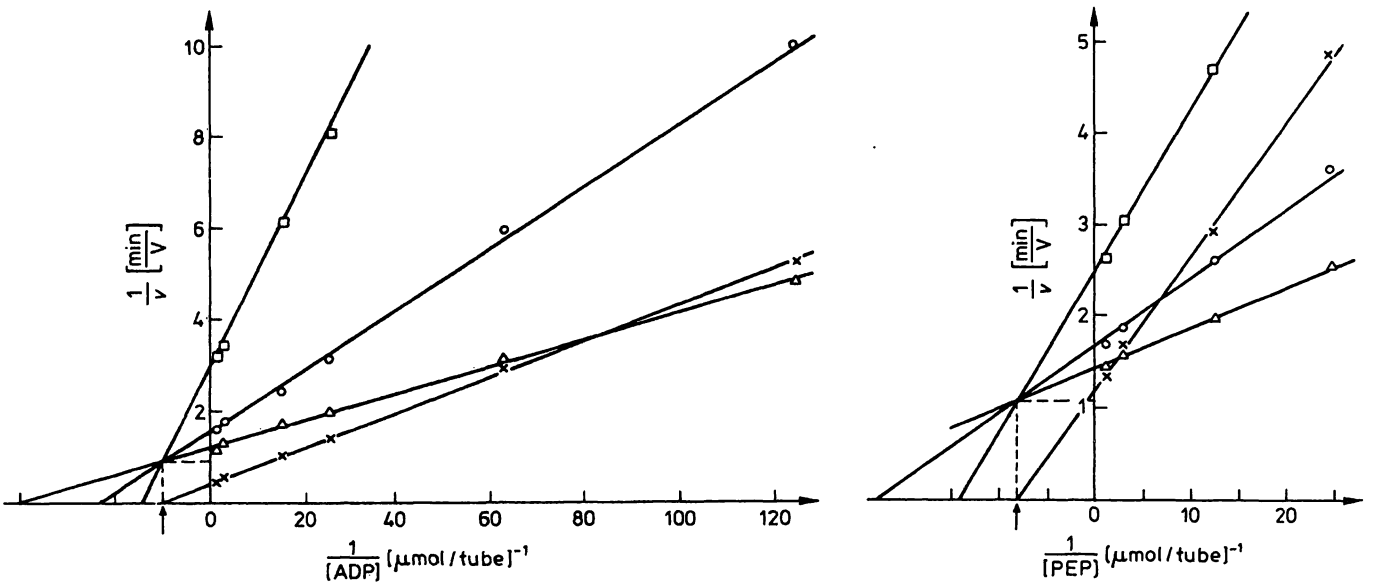

Fig. 4. The *Lineweaver-Burk* plot used to determine the K_m value for conjugated pyruvate kinase, compared with the K_m value for the nonconjugated enzyme (x—x). The arrow on the abscissa represents the K_m value.
a) K_m-values for ADP
b) K_m-values for phosphoenol pyruvate (PEP). Molar ratios enzyme: coupling reagent succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate:
1:1.2 □—□ 1:6 ○—○ 1:10 △—△

Chemiluminescent conjugates

Figure 5 shows the relative light intensity generated by diazoluminol, diazoisoluminol and N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide under identical conditions. The detection limit of diazoisoluminol is around 3 decades higher than that for diazoluminol. The detection limit of (N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide is similar to that for diazoluminol.

The results from the preparation of antibody conjugates using diazoluminol and diazoisoluminol agreed in parts with other studies (10), however differ in that in this laboratory mainly diazoluminol, and not diazoisoluminol, conjugates have been found to be more suitable. Diazoisoluminol gave no useable conjugates with antibodies at the pH and incubation times tested here.

Figure 6 shows the effect of testing the binding of the column eluates of diazoluminol and diazoisoluminol donkey-anti-rabbit IgG conjugates to rabbit IgG coated polystyrene balls (= B_0 or specific binding) and to transferrin-coated balls as protein-coated balls not containing rabbit IgG (= UB or unspecific binding) under identical conditions namely: 2 μl eluate, 300 μl incubation buffer and a coated polystyrene ball were incubated at ambient temperature for 1 h,

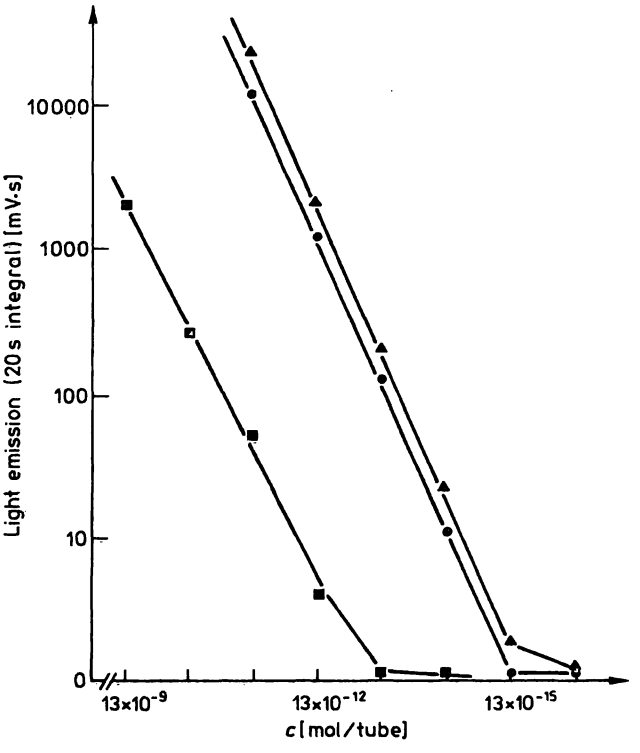

Fig. 5. The light output obtained from different concentrations of diazoluminol (●—●), diazoisoluminol (■—■) and N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide (▲—▲). The concentrations (mol/tube) of the test substances are shown on the abscissa. The values on the ordinate represent the area under the light-emission curve integrated over 20 seconds.

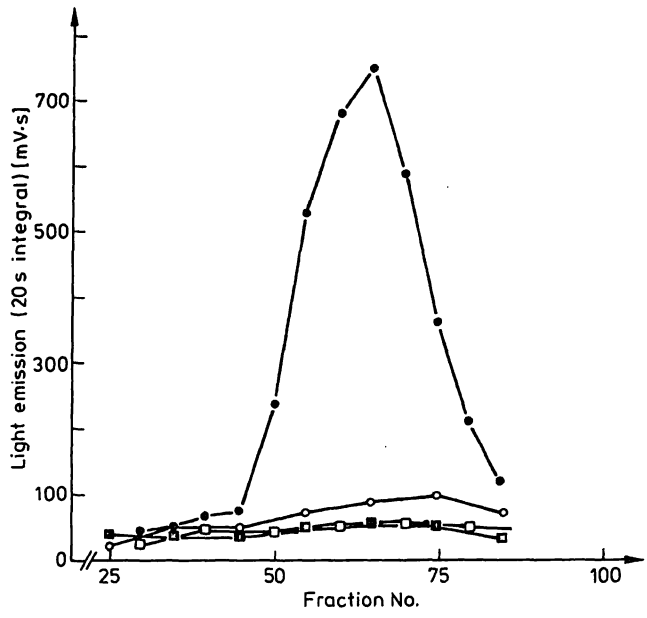

Fig. 6. Binding properties of diazoluminol-IgG and diazoisoluminol-IgG in fractions from an Ultrogel A4 column. 5 µl/fraction were incubated with a rabbit IgG coated polystyrene ball (B_0, closed symbols) and a human transferrin coated ball (UB, symbols open) to determine specific and unspecific binding respectively.
Circles: diazoluminol-IgG
Squares: diazoisoluminol IgG

washed with 1 × 1 ml incubation buffer and 1 × 1 ml 0.15 mol/l NaCl, then transferred to the luminometer where the light emission was initiated.

As can be seen, the diazoisoluminol conjugate shows a much lower specific binding than the diazoluminol conjugate, showing that isoluminol derivatives could not be used in that way in any assay systems.

At pH 4 diazoluminol gave no useful conjugates. When using diazoluminol a reaction time of 24 h at 4 °C gave optimal results. Shorter incubation times gave a lower substitution rate, whereas incubation times longer than 24 h did not give rise to better conjugates.

Table 3a shows the effect of the substitution ratio of diazoluminol:donkey anti rabbit IgG, the optimal substitution rate being around 40:1 as far as the signal to noise ratio (B_0/UB) was concerned.

Figure 7 shows the effect of the molar ratio diazoluminol:IgG on the binding characteristics of the Ultrogel A4 column fractions. 10 µl per fraction were tested analogically to the conditions described above for figure 6.

It can be seen that a higher substitution rate only led to a dramatic increase in the unspecific binding as can be seen in the 1:100 curve.

Tab. 3a. Comparison between the maximal (B_0) and unspecific binding (UB) of different diazoluminol IgG conjugates represented by the fraction from the Ultrogel A4 column eluate with maximal binding.

Ratio IgG to diazoluminol	B_0 (mV · s)	UB (mV · s)	Ratio UB/B_0
1:8	823	52	0.063
1:20	3310	66	0.019
1:40	13440	220	0.016
1:100	9840	465	0.047
1:200	9715	975	0.100

All measurements performed on an LKB 1251 Luminometer.
Assay conditions:
For B_0 – rabbit IgG coated polystyrene balls
For UB – human transferrin coated balls
10 µl column eluate, 300 µl incubation buffer and 1 coated polystyrene ball were incubated for 60 min at ambient temperature, washed with 1 × 1 ml incubation buffer containing 0.5 ml/l Tween 20, followed by 1 × 1 ml 0.15 mol/l NaCl.

Measuring conditions:
Transfer ball to measuring cuvette.
Light reaction initiated with 200 µl 0.8 mol/l NaOH, 100 µl microperoxidase – MP 11 (5 µmol/l), 120 µl H_2O_2 (0.3 ml/l).
Integration time 20 seconds, results given as millivolt · seconds (mV · s).

Table 3b shows the comparison between maximal (B_0) and unspecific binding (UB) for different N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester donkey anti rabbit IgG conjugates using different substitution ratios.

A substitution ratio of about 60:1 gave the best results, a higher substitution of 120:1 giving a higher noise to signal ratio (UB/B_0). Lower substitution ratios gave a lower light intensity but no difference in the ratio UB/B_0.

Tab. 3b. Comparison between the maximal (B_0) and unspecific binding (UB) of different (N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide) active ester donkey anti-rabbit IgG conjugates using the fraction from the Ultrogel A4 column with maximal binding.

Ratio IgG to N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester	B_0 (mV · s)	UB (mV · s)	Ratio UB/B_0
1: 30	6085	192	0.031
1: 60	21662	686	0.032
1:120	33442	1706	0.051

Assay conditions:
5 µl of a 1:10 dilution (in elution buffer) of the fraction with maximal binding were used analogically to the assay conditions described in table 3a.

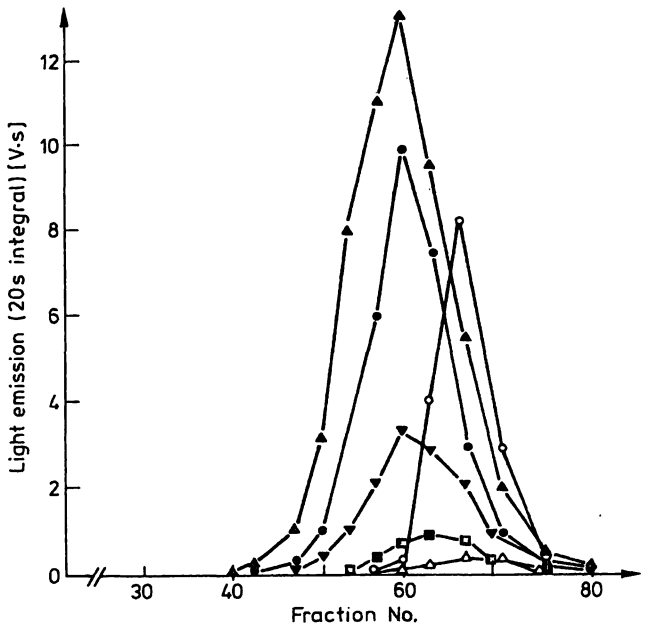

Fig. 7. Effect of different substitution rates diazoluminol: IgG on binding profile of column eluent. The labelled antibody was as in figure 6, i.e. an anti rabbit IgG, the coated balls for specific (B_0) and unspecific binding (UB) again being rabbit-IgG and human transferrin as above. The incubation time was 60 min, 5 µl of the column fraction being tested. The UB represents the non-specific adsorption to or reaction with the transferrin coated polystyrene ball.

	Ratio IgG: diazoluminol	
B_0 ●—●	1:100	○—○ UB
B_0 ▲—▲	1: 40	△—△ UB
B_0 ▲—▲	1: 20	
B_0 ■—■	1: 8	

Discussion

The problems and possible solutions for set up solid phase based luminescence immunoassays have been limited to the preparation of luminescence conjugates and hapten-protein conjugates in the second part of this trilogy.

The bioluminescent labels here described show the problems of working with kinases, that is the coupling of kinases to antigens or antibodies without loss of antigenic or enzymatic activity. Of all kinases tested only pyruvate kinase was robust enough to "survive" coupling using the heterobifunctional reagents tested here. The other kinases, although attractive on paper, for example adenylate kinase with its molecular weight of 21000, resistance to acid pH, high turnover rate and single substrate (ADP) were unable to be used due to their sensitivity to maleimide. Other coupling reagents (carbodiimides, pentane-1,5-dial, bisepoxirane) were found to be unsuitable as the enzyme activity disappeared during coupling. The results show that pyruvate kinase, despite its high molecular weight (240000) was used because of available SH-groups not present in the active centre.

Whether the use of SH-group introducing reagents such as (N-succinimidyl 3-(2-pyridyldithio) propionate) could make the other kinases useable, is a point worth considering.

The results from the *Lineweaver-Burk* plots confirmed that the coupling reagents here chosen led to changes in enzyme activity and structure, as measured by substrate affinity. As previously stated (1), difficulties of finding a suitable antimicrobial reagent for bioluminescent labels of this type presents a major drawback, which suggests that such labels should only be used where insufficient sensitivity can be obtained with chemiluminescent labels. The latter do not suffer such problems, partly due to their hydrazide structure in the case of diazoluminol, which in itself appears to exert bacteriostatic properties.

Many of the chemiluminescent labels described in the literature suffer from the drawback that their synthesis is somewhat complicated (6). The labels described here are simple to produce, and do not require much expertise and equipment, as well as giving out sufficient light for most purposes, although N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide gave the best results.

Diazoluminol conjugates have in contrast to diazoisoluminol conjugates a higher light output (10). The reaction of diazoisoluminol in the presence of proteins was far quicker than of diazoluminol, as monitored by the decolorisation. This may be explained by the active diazogroup, which in isoluminol is located on the less sterically hindered 6 (4)-position. Diazoluminol is not as reactive due to the position of the oxo-group on the azine ring, and due to its ability to build a stable internal structure (see fig. 8). The reaction of proteins with diazoluminol could be divided into two distinct phases, a rapid reaction with the protein in alkaline solution (pH 9—10) in which the reaction mixture remained red (excited electrons in the diazo bridge are still present). Such conjugates are stable enough to be separated on an agarose gel column (Ultrogel A4 or A6), however they are unstable enough to transfer the diazoluminol group to another protein if the latter is present in excess. At this stage a transitional bonding is present in which both ionic and covalent components are present. The second stage of the reaction is slower and is associated with a disappearence of the red coloration, and the appearance of a yellow-orange colour, in which the covalent bonding is stable. These conjugates can be stored at −20 °C for over a year without appreciable loss in immunological and luminogenic activity.

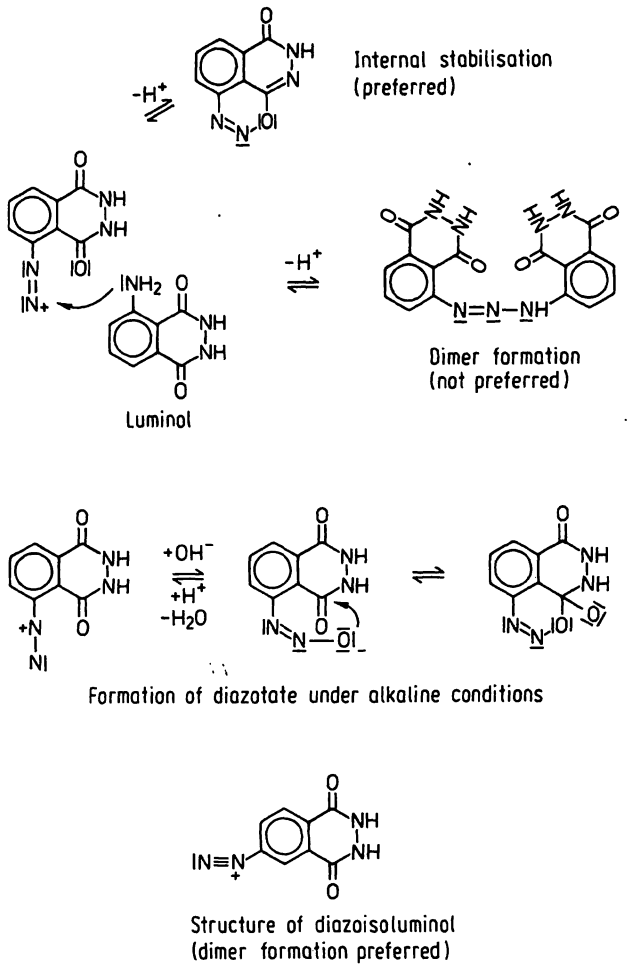

Fig. 8. This set of reactions shows the possible reactions occurring during diazotisation under alkaline conditions, as carried out here. Diazoluminol prefers an internal stabilisation (above reaction sequence – centre picture) whereas diazoisoluminol prefers dimer formation as the position of the diazogroup is unfavorable for an internal stabilisation. This may account for the fact that diazoisoluminol is formed in solution, but precipitates out within 30 minutes as a golden-yellow crystalline deposit. Diazoluminol at equimolar concentrations remains in solution. The middle set of reactions shows the formation of diazotate under alkaline conditions.

An important point to mention here, is that the materials to be diazotised must be free from azide, as diazoluminol reacts with sodium azide to form compounds which do not react with the tyrosine moieties of the protein. Here the decolorisation of the reaction mixture takes place within seconds, a yellow solution being formed. If this solution is then adjusted to pH 7 with phosphate buffer, it darkens due to reaction with atmospheric oxygen, suggesting that poly-phenol derivatives may be formed. This would be in accordance with a free radical reaction following the loss of nitrogen from the diazoluminol-azide complex. These unwanted effects can be avoided by treatment of the material to be diazotised with DEAE cellulose in the form of a short column (2, 15), as already described.

The comparison of the detection limits for diazoluminol and N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide shows no significant difference. On the other hand N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester labelled IgG-molecules show much better light intensities than those labelled with diazoluminol. This could be explained by the different reaction mechanism of these two compounds. The reaction of diazoluminol with tyrosyl-residues of IgG is of an oxidative nature and may destroy antibody structure (compare chloramine-T iodination of certain proteins which lead to activity loss). The N-(4-aminobutyl)-N-ethylisoluminol hemisuccinamide active ester reacts with amino-moieties under non-oxidative conditions (compare *Bolton-Hunter* iodination of proteins).

Finally, it can be stated that the choice of label used, i.e., whether chemi- or bioluminescent depends to a large extent upon the sensitivity of the assay, and although the assays described to date mainly use the former (7, 8, 9), the latter may have to be used until sufficient sensitivity can be achieved using chemiluminescent markers.

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